Characterization of the Anti-LAG-3 Antibody REGN3767

Preclinical Development of the Anti-LAG-3 Antibody REGN3767: Characterization and Activity in Combination with the Anti-PD-1 Antibody Cemiplimab in Human PD-1xLAG-3 Knock-In Mice

Elena Burova, Aynur Hermann, Jie Dai, Erica Ullman, Gabor Halasz, Terra Potocky, Seongwon Hong, Matt Liu, Omaira Allbritton, Amy Woodruff, Jerry Pei, Ashique Rafique, William Poueymirou, Joel Martin, Douglas MacDonald, William Olson, Andrew Murphy, Ella Ioffe, Gavin Thurston, and Markus Mohrs¹

Regeneron Pharmaceuticals, Inc., Tarrytown, NY 10591

¹Corresponding author: Markus Mohrs, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591. Phone: 914-847-1122; Fax: 914-847-7453;
E-mail: markus.mohrs@regeneron.com

Running title: Characterization of human anti-LAG-3 antibody REGN3767

Keywords: LAG-3, Checkpoint blockade, Monoclonal antibody, Immunotherapy, Cancer

Disclosure of Potential Conflicts of Interest

All authors are employees of Regeneron Pharmaceuticals, but otherwise have no conflicts of interest to declare.

Financial support statement

This work is funded by Regeneron Pharmaceuticals, Inc.

Electronic work count

Abstract: 228

Words of text: 4684

Number of figures and tables

Figures: 6
ABSTRACT

In the tumor microenvironment, multiple inhibitory checkpoint receptors can suppress T cell function, thereby enabling tumor immune evasion. Blockade of one of these checkpoint receptors, PD-1, with therapeutic antibodies (Abs) has produced positive clinical responses in various cancers, however, the efficacy of this approach can be further improved. Simultaneously targeting multiple inhibitory checkpoint receptors has emerged as a promising therapeutic strategy. Here we report the development and characterization of REGN3767, a fully human IgG4 Ab targeting LAG-3, another inhibitory receptor on T cells. REGN3767 binds human and monkey LAG-3 with high affinity and specificity and blocks the interaction of LAG-3 with its ligand, MHC class II. In an engineered T cell/antigen-presenting cell (APC) bioassay, REGN3767 alone, or in combination with cemiplimab (REGN2810, human anti-PD-1 Ab), blocked inhibitory signaling to T cells mediated by hLAG-3/MHCII in the presence of PD-1/PD-L1. To test the in vivo activity of REGN3767 alone or in combination with cemiplimab, we generated human PD-1xLAG-3 knock-in mice, in which the extracellular domains of mouse Pdcd1 and Lag3 were replaced with their human counterparts. In these humanized mice, treatment with cemiplimab and REGN3767 showed increased efficacy in a mouse tumor model and enhanced the secretion of proinflammatory cytokines by tumor-specific T cells. The favorable pharmacokinetics and toxicology of REGN3767 in non-human primates, together with enhancement of anti-tumor efficacy of anti-PD-1 Ab in preclinical tumor models, supports its clinical development.
INTRODUCTION

Therapeutic monoclonal antibodies targeting inhibitory receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell death 1 (PD-1) show impressive clinical activity with an acceptable benefit risk ratio in several tumor types (1-4). However, sustained responses are only achieved in a minority of patients, suggesting that combination approaches may be required to overcome tumor immune escape mechanisms (5). Antibodies blocking the interaction of the inhibitory receptor lymphocyte-activation gene 3 (LAG-3) with its ligand, major histocompatibility complex class II (MHC class II), may invigorate the immune response to cancer, especially in combination with antibodies blocking the PD-1/PD-L1 axis (reviewed in 6-8).

The inhibitory receptor LAG-3, a CD4-like molecule, is expressed on activated CD4+ and CD8+ T cells, a subset of regulatory T cells (Tregs), natural killer cells, B cells and plasmacytoid dendritic cells (8-11). LAG-3 suppresses T cell activation, proliferation and homeostasis of T cells (11, 12) and has been reported to play a role in Treg suppressive function (13). LAG-3 binds to MHC class II, which is expressed on dendritic cells, macrophages, B cells and epithelial cancer cells, and serves to present peptides to CD4+ T cells (14-16). LAG-3 has been shown to be expressed on dysfunctional T cells in chronic viral infections (17, 18) and during tumor progression in cancer patients (19, 20). CD8+ T cells co-expressing PD-1 and LAG-3 display severely impaired effector functions in a murine model of self-antigen tolerance (21), and antigen-specific T cells in human ovarian cancer are negatively regulated by PD-1 and LAG-3 (20). Multiple reports demonstrate that a large fraction of PD-1 expressing CD8+ and CD4+ tumor infiltrating lymphocytes (TILs) co-express LAG-3, supporting the dominance of PD-1 in modulating anti-tumor T cell responses, as well as a direct role of LAG-3 in further suppressing the activity of a subset of
Characterization of the Anti-LAG-3 Antibody REGN3767

PD-1 expressing T cells (18, 20-24). Mice deficient for both PD-1 and LAG-3 show spontaneous autoimmunity and lethality, not seen in either single knockout (22, 25). Additionally, dual blockade of PD-1 and LAG-3 with antibodies synergistically inhibits tumor growth in preclinical tumor models (22, 23, 26). Thus, it stands to reason that antagonistic anti-LAG-3 antibodies may improve efficacy in combination with anti-PD-1 therapies in the clinic (7, 8, 27-30). Currently, there are several LAG-3-targeting treatments in various phases of clinical development (31-34). In particular, combination therapy of anti-LAG-3 (BMS-986016) plus anti-PD-1 (nivolumab) has shown promising clinical efficacy in melanoma patients (33, 34). We have previously generated cemiplimab, a human anti-PD-1 antibody blocking PD-1/PD-L1 mediated T cell inhibition (35). Here we describe the generation of REGN3767, a fully human high-affinity anti-LAG-3 antibody, using VelocImmune mice containing human immunoglobulin gene segments (36, 37). In vitro, REGN3767 binds LAG-3 with high affinity and blocks LAG-3/MHC class II-driven T cell inhibition. In vivo, REGN3767 enhances the anti-tumor activity of the anti-human PD-1 antibody cemiplimab against syngeneic colorectal carcinomas in mice humanized for the extracellular domains of PD-1 and LAG-3. The preclinical results presented here supported the initiation of clinical trials using REGN3767 in monotherapy or in combination with cemiplimab in patients with solid tumors.

MATERIALS AND METHODS

Antibodies

To generate anti-human LAG-3 antibodies, VelocImmune mice, carrying genes encoding human immunoglobulin heavy and kappa light chain variable regions (36, 37), were immunized with recombinant human LAG-3-mFc protein (Regeneron), containing the
Characterization of the Anti-LAG-3 Antibody REGN3767

extracellular domain of LAG-3 (amino acids 1-450) and the Fc portion of mouse IgG2a. Hybridomas were generated from splenocytes and supernatants were screened for binding to HEK293 cells transfected with human LAG-3. REGN3767 was engineered as a human IgG4 containing a serine to proline substitution (S228P) in the hinge region to minimize half-antibody formation (38). To reduce binding to Fcγ receptors (FcγR), REGN3767 also contains 3-amino-acid substitutions (P236VA238) derived from the lower hinge region of IgG2 replacing 4 amino acids (E236FLG239) in the corresponding lower hinge region of IgG4 (39). The amino acid sequences of the heavy and light chains of REGN3767 (Genebank accession numbers: MN200290 and MN200291) are shown in Supplementary Fig. S1. The hIgG4 (S228P) anti-human PD-1 antibody cemiplimab (REGN2810), that binds to both human and monkey PD-1 proteins and blocks PD-1 interactions with PD-L1 and PD-L2, was described previously (35).

REGN3767 binding to human LAG-3 proteins

Binding kinetics of REGN3767 mAb to LAG-3 proteins at 25°C and pH 7.4 was measured in Surface Plasmon Resonance (SPR)-Biacore studies by first capturing REGN3767 on a CM5 (Biacore) surface chip pre-coated with anti-human kappa goat polyclonal F(ab’)2 Ab (GE Life Sciences) and then injecting various concentrations of LAG-3 proteins over the surface. Following capture, hLAG-3.mmH (myc-myc-polyhistidine tag), rLAG-3.mmH, or mLAG-3.mmH at concentrations ranging from 50 nmol/L to 0.78 nmol/L, or hLAG-3.hFc protein ranging from 25 nmol/L to 0.39 nmol/L were individually injected over the REGN3767-captured surfaces. The kinetic parameters were obtained by globally fitting the data to a 1:1 binding model using Biacore T200 Evaluation.
Characterization of the Anti-LAG-3 Antibody REGN3767

Cell-cell adherence assay

The ability of REGN3767 to block the binding of hLAG-3 to human MHC II-positive (Raji) B cells or murine MHC II-positive (A20) B cells was assessed using a cell-cell adherence assay. Fluorescently labeled Raji or A20 cells were examined for adherence to HEK293/hLAG-3 cells in the presence or absence of REGN3767. The parental HEK293 cell line has no detectable expression of human LAG-3 as determined by flow cytometry. To confirm expression of MHC II, Raji cells were stained with anti-human HLA-DR (clone Tu36, BD Biosciences) and A20 cells were stained with anti-mouse I-A/I-E (clone M5/114.15.2, Biolegend). Raji or A20 cells were labeled with Calcein AM (Life Technologies). Human Fc block (BD Pharmingen) was added to pre-labeled Raji cells and mouse Fc block (BD Pharmingen) was added to A20 cells at a final concentration of 10 μg/mL. HEK293 parental and HEK293/hLAG-3 cells were added at 1.2 x 10^4 cells/100 μL into 96-well plates and cultured overnight. REGN3767 or the isotype matched control antibody REGN2759 (Regeneron) were added at increasing concentrations for 1 hour, followed by incubation with 1.2x10^5 labeled Raji or A20 cells for 1 hour. Non-adherent cells were removed by washing. Relative florescence units (RLU) of adherent labeled Raji or A20 cells were measured at an excitation/emission wavelength of 485nm/535nm on a VICTOR X5 plate reader.

Generation of human PD-1xLAG-3 knock-in mice

VelociGene technology was used to generate human PD-1xLAG-3 knock-in mice as described previously (40). Briefly, a Lag3 targeting vector was engineered that replaced 1750 bp of the extracellular portion of the mouse Lag3 gene (containing exons 2-4) with the corresponding 1741 bp region of the human gene (exons 2-4). Dual humanized mouse
Characterization of the Anti-LAG-3 Antibody REGN3767

Embryonic stem (ES) cells were created by electroporation of the Lag3 targeting vector into C57BL/6N mouse ES cells that contained a humanized Pdcd1 gene encoding the extracellular portion of human PD-1 and the transmembrane and intracellular portion of mouse Pdcd1 (35). Correct gene targeting in ES cell clones was identified by a loss of allele assay (41). Dual humanized ES cell clones were used to implant female mice to generate a litter of pups containing both humanized genes (i.e., Lag3 and Pdcd1).

**Tumor challenge experiments**

MC38.Ova cells were previously described (35) and authenticated by short tandem repeat profiling in 2016 (IDEXX BioResearch). MC38.Ova cells (5x10^5) were injected subcutaneously (s.c.) in the hind flank of 8-10 weeks old female human PD-1xLAG-3 knock-in mice. Tumors were measured semiweekly using a caliper and reported as mm^3 (length x width^2/2). In a prophylactic tumor model, antibodies were administered intraperitoneally (i.p.) in 200 μl starting on day 3 after tumor implantation, and then twice a week for two weeks. In an established tumor model, mice were randomized on day 10-11 after implantation when tumors reached 100 mm^3. Mice were treated with antibodies on the randomization day and then twice a week for two weeks. Mice were euthanized when the tumor volumes reached 2000 mm^3 or when tumors ulcerated. The protocol was approved by the Regeneron Pharmaceuticals Institutional Animal Care and Use Committee (IACUC).

**Flow cytometric assessment of tumoral T-cell activation**

Tumors were harvested and processed using GentleMacs cell disruptors (Miltenyi) to generate single cell suspensions. Lymphocytes were enriched by Percoll (GE Healthcare) gradient centrifugation, stained with fluorescently-labeled antibodies and analyzed on a
Characterization of the Anti-LAG-3 Antibody REGN3767

FACS Canto flow cytometer (BD Biosciences). *Ex vivo* TILs stimulation was performed with 1 μg/ml Ova257-264 MHC I peptide and 5 μg/ml Ova323-339 MHC II peptide (InvivoGen) in the presence of Brefeldin A (1 μg/ml) for 4 hours at 37°C. Draining lymph nodes (dLNs) were processed into single cell suspension by mechanical dissociation. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) and ionomycin (500 ng/ml) for 4 hours in the presence of Brefeldin A (1 μg/ml), and then processed with the Cytofix/Cytoperm kit (BD Biosciences) for intracellular cytokine staining. The following Ab clones were used: mCD45 (30-F11, BioLegend), mCD4 (GK1.5, BioLegend), mCD8a (53-6.7, BioLegend), mIFNγ (XMG1.2, eBioscience), mTNFα (MP6-XT22, Biolegend), hPD-1 (EH12.2H7, BioLegend), hLAG-3 (3DS223H, eBioscience), mouse PD-1 (J43, eBioscience), mouse LAG-3 (C9B7W, BioLegend).

**Cytokine quantification in mouse serum and in spleen**

Spleens were harvested from tumor bearing mice and manually dissociated into single-cell suspensions. Spleen homogenates were clarified by centrifugation and the protein content was quantified using a BCA assay (Pierce). 50 μg of supernatant from a spleen sample and 25 μl of serum were used in duplicates to measure cytokine concentrations by the V-PLEX Proinflammatory Panel 1 mouse kit according to the manufacturer’s instructions (Meso Scale Discovery).

**Pharmacokinetics studies in cynomolgus monkeys**

Female cynomolgus monkeys (5 animals/dose group) received a single i.v. infusion (1, 5, or 15 mg/kg) or s.c. injection (1 or 15 mg/kg) of REGN3767. Blood samples were collected for measurement of functional REGN3767 concentrations and anti-drug antibody (ADA) in...
Characterization of the Anti-LAG-3 Antibody REGN3767

serum at predose and post-dose at various times for up to 56 days. Concentrations of functional REGN3767 in serum were determined using an enzyme-linked immunosorbent assay (ELISA). This procedure employed microtiter plates coated with a recombinant protein containing the extracellular domain of human LAG-3 and utilized REGN3767 as the reference standard. REGN3767 captured on the plate was detected using a biotinylated mouse anti-human IgG4 mAb REGN1298 (Regeneron), followed by NeutrAvidin conjugated with horseradish peroxidase.

RESULTS

In vitro characterization of REGN3767 as a single agent and in combination with anti-PD-1 antibody cemiplimab.

Clone REGN3767 was selected from a panel of human antibodies generated by immunization of VelocImmune mice transgenic for human variable regions (36, 37). REGN3767 bound specifically to human LAG-3, with a $K_D$ of 3.22 nmol/L and 0.13 nmol/L for monomeric and dimeric recombinant proteins, respectively. No cross-reactivity to mouse and rat LAG-3 was detected, consistent with low amino acid sequence identity between the extracellular regions of human and mouse (70%) or rat (67%) LAG-3. REGN3767 bound HEK293 cells engineered to overexpress human or cynomolgus monkey LAG-3 with a similar EC$_{50}$ value of 1.1 nmol/L and 0.8 nmol/L, respectively (Fig. 1A). Flow cytometry confirmed REGN3767 binding to LAG-3 on activated primary human and cynomolgus monkey CD4$^+$ and CD8$^+$ T cells (Supplementary Fig. S2A and S2B). REGN3767 bound activated CD8$^+$ T cells, expressing either low or high level of early activation marker CD69, with comparable EC$_{50}$ values of 0.65-1.3 nmol/L and 0.7-1.0
Characterization of the Anti-LAG-3 Antibody REGN3767

nmol/L for human and cynomolgus monkey donors, respectively (Supplementary Fig. S2C and S2D). The staining intensity was lower on activated CD4+ T cells compared to CD8+ T cells in both species, suggesting lower LAG-3 expression; this observation is in line with published studies (42, 43). In summary, REGN3767 exhibited similar EC50 values for binding to human and cynomolgus monkey LAG-3 overexpressed on HEK293 cells or expressed endogenously on activated primary cells.

Human LAG-3 binds both human and mouse MHC II (15, 16), so the ability of REGN3767 to block these interactions was assessed using a cell-cell adherence assay. A similar assay was previously used to demonstrate MHC II interactions with CD4 (14) and to identify the domains of LAG-3 responsible for binding to MHC II (16). REGN3767 blocked the binding of MHC II-positive human Raji B cells and murine A20 B cells, respectively, to HEK293/hLAG-3 cells with EC50 values of 4.2 nmol/L and 7.1 nmol/L, respectively (Fig. 1B). Binding of HEK293/hLAG-3 to Raji cells or A20 cells was reversed when anti-MHC class II antibody (anti-human HLA-DR or anti-mouse HLA I-A/I-E, respectively) was added to the system (Supplementary Fig. S3), suggesting that MHC class II plays a major role in this process.

Neither Raji nor A20 cells displayed significant adherence to parental HEK293 cells, demonstrating that LAG-3 expression on HEK293 is required for adherence.

The potential of REGN3767 in combination with cemiplimab to rescue T cell activity was evaluated in in vitro functional assays. In an engineered T cell/APC luciferase-based bioassay (Supplementary Fig. S4, Supplementary Materials and Methods), activation of engineered JRT3.T3.5 T cells is driven by engagement of a TCR (0b2F3) with its cognate peptide MBP85-99 presented on MHC II by engineered HEK293 cells that serve as APCs (44). JRT3.T3.5 T cells were additionally engineered to express chimeric human LAG-3 and
Characterization of the Anti-LAG-3 Antibody REGN3767

human PD-1. Chimeric human LAG-3 was constructed of the human LAG-3 extracellular domain and the cytoplasmic domain of the human inhibitory receptor CD300a, which contains immunoreceptor tyrosine-based inhibition motifs (ITIM). APC express human MHC II with or without exogenously expressed human PD-L1. In both T cell/APC formats, REGN3767 was titrated in the presence of 30 nmol/L of cemiplimab. In the absence of PD-1/PD-L1 signaling, REGN3767 restored T cell activity with an EC$_{50}$ value of 4.7 nmol/L; the activity was unaffected by the presence of cemiplimab, as expected. When the bioassay included the PD-1/PD-L1 signaling axis, REGN3767 increased T cell activation when combined with cemiplimab.

In an additional in vitro functional assay of allogeneic T cells: PBMC mixed lymphocyte reaction (MLR), cemiplimab and REGN3767, used as single agents augmented IFN$\gamma$ release by the alloreactive T cells (Fig. 1C). Combination of cemiplimab and REGN3767 further enhanced T-cell reactivity in the presence of a T-cell receptor stimulus. To show that REGN3767 alone, or in combination with cemiplimab, does not induce nonspecific T cell activation, we performed proliferation and cytokine release assay in PBMC ex vivo. The addition of cemiplimab and/or REGN3767 had no effect on PBMC proliferation and did not induce significant production of IFN$\gamma$, TNF$\alpha$, IL-2 and IL-10 from freshly isolated or pre-activated PBMC. Lastly, we tested the ability of REGN3767 to induce antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) in vitro. REGN3767 at concentrations ranging from 95 fmol/L to 100 nmol/L did not mediate ADCC or CDC activity against Jurkat/hLAG-3 target cells in the presence of human NK cells or human serum complement, respectively. Taken together, these data suggest that
REGN3767 and cemiplimab treatments could synergistically enhance effector T cell responses without nonspecific T cell activation.

**REGN3767 enhances the anti-tumor activity of cemiplimab in human PD-1xLAG-3 knock-in mice by promoting T cell mediated immunity**

We tested the anti-tumor activity of REGN3767 as a single agent or in combination with Regeneron’s clinical anti-PD-1 antibody cemiplimab in a mouse MC38.Ova colon carcinoma model. Because cemiplimab and REGN3767 do not cross-react with mouse PD-1 and LAG-3, respectively, we genetically engineered dual human PD-1xLAG-3 knock-in mice to express the extracellular domains of human PD-1 and human LAG-3 as chimeric proteins containing the corresponding mouse transmembrane and cytoplasmic domains from the respective mouse loci (Supplementary Fig. S5A). As shown in Supplementary Fig S5B, PD-1xLAG-3 dual knock-in mice faithfully express human PD-1 and LAG-3 proteins on activated T cells. The absence of staining with anti-mouse LAG-3 and anti-mouse PD-1 antibodies confirmed that full-length mouse PD-1 and LAG-3 were deleted in PD-1xLAG-3 dual knock-in mice. Of note, as human PD-1 binds both human and mouse PD-L1 with similar affinity, and human LAG-3 binds both human and mouse MHC II (45, 15, 16), humanization of PD-L1 and MHC II are not required. Homozygous human PD-1xLAG-3 dual knock-in mice displayed a normal life span and no overt signs of autoimmunity for at least one year, whereas PD-1xLAG-3 knockout mice develop strain and tissue-specific autoimmunity (22, 25).

In MC38.Ova tumors in PD-1xLAG-3 knock-in mice, PD-1 and LAG-3 co-expression was largely limited to CD4+ and CD8+ TILs, while splenic T-cells predominantly expressed PD-1 but not LAG-3 (Supplementary Fig. S5C), consistent with previous reports (22).
Characterization of the Anti-LAG-3 Antibody REGN3767

Approximately 47% of CD8+ and 50% of CD4+ TILs cells were double positive, supporting the hypothesis that these two receptors could directly cooperate in T cell suppression.

In a prophylactic MC38.Ova tumor model, monotherapy with cemiplimab at 10 mg/kg was moderately efficacious, while REGN3767 dosed at 25 mg/kg had a minimal effect as a monotherapy (Fig. 2A and 2C). However, combination treatment prolonged survival (p<0.0001) compared to isotype antibody control treatment at the end of the study on day 46. Five out of 12 mice were tumor-free in the combination treatment group, whereas only 1 out of 12 and 2 out of 12 mice were tumor-free in REGN3767 and cemiplimab monotherapy groups, respectively. In a therapeutic model, treatment of 100 mm³ MC38.Ova tumors with cemiplimab at 10 mg/kg was partially efficacious, while there was no single agent activity of REGN3767 at 25 mg/kg (Fig. 2B and 2D). Combination therapy showed a significant reduction of tumor growth on day 22 relative to isotype control (p<0.05) and to REGN3767 (p<0.01) groups.

To determine the relative contribution of cemiplimab and REGN3767 to the combination effect, we tested regimens, in which the dose of one antibody was constant and the other was titrated (Supplementary Table S1). When the anti-PD-1 antibody cemiplimab was administered at 10 mg/kg (5 injections within 2 weeks), addition of REGN3767 at increasing doses (5 mg/kg or 25 mg/kg) on the same schedule enhanced efficacy. Similarly, when REGN3767 was dosed at 25 mg/kg, lowering cemiplimab dose from 10 mg/kg to 1 mg/kg reduced the anti-tumor activity. Thus, the co-blockade of PD-1 and LAG-3 is superior to either monotherapy and the efficacy is more dependent on anti-PD-1 activity.

Next, we determined the impact of anti-PD-1 and anti-LAG-3 treatment on T cells in MC38.Ova tumors. Upon treatment of mice with 100 mm³ MC38.Ova tumors with a combination of cemiplimab and REGN3767 the average tumor size was significantly
Characterization of the Anti-LAG-3 Antibody REGN3767

smaller than in the isotype control or anti-LAG-3 treated groups (Fig. 3A), and combination treatment, but not monotherapy, resulted in significant increase of tumor infiltrating CD4+ T cells (Fig. 3B). To examine antigen-specific T cells in tumors, TILs were re-stimulated ex vivo with OVA MHC I and MHC II peptides followed by intracellular staining for IFNγ and TNFα. Neither mono- nor combination therapy had a significant impact on the frequency of IFNγ or TNFα producing CD8+ TILs, however, the frequency of IFNγ and TNFα producing CD4+ TILs was significantly higher in cemiplimab and dual-antibody treated groups as compared to the control treatment (Fig. 3C). As T cell priming occurs in tumor draining lymph nodes (dLN), we examined antigen specific and IFNγ expressing T cells in draining lymph nodes. There was no expansion of OVA-specific CD8+ T cells in draining LN as a result of single or dual anti-PD-1 and anti-LAG-3 treatment. However, we observed a significant increase in IFNγ producing CD4+ and CD8+ T cell in dLN in the dual anti-PD-1 and anti-LAG-3 treatment group (Fig. 3D). In contrast to tumors, there were no changes in TNFα production by T cells in response to single or dual antibody therapy in dLN. Taken together, these data suggest that cemiplimab and REGN3767 combination immunotherapy reduces tumor growth by increasing the proportion of effector T cells both in the tumor and in draining lymph nodes. In addition, we detected increased concentrations of IFNγ, TNFα and IL-10 in serum and spleen lysates of PD-1xLAG-3 knock-in mice treated with both REGN3767 and cemiplimab compared to treatment with either antibody alone (Fig. 4).

To explore the underlying molecular mechanisms of MC38.Ova tumors response to anti-PD-1 and anti-LAG-3 treatment we conducted RNA sequencing of MC38.Ova tumors in PD-1xLAG-3 mice following treatment with one or two doses of cemiplimab, REGN3767, or their combination. Mice were randomized on day 10 after MC38.Ova cells implantation when tumors reached 100 mm³. Treatment with two doses of REGN3767, cemiplimab or
their combination (on randomization day 10 and on day 14) significantly reduced tumor volumes compared to isotype control treatment, while single dose treatment on day 10 did not show anti-tumor activity (Fig. 5A). Both REGN3767 and cemiplimab promoted robust transcriptional changes in tumors, and combined PD-1 and LAG-3 blockade resulted in enhanced immune activation signatures. The combination treatment resulted in significant upregulation of 1616 and 2256 genes after one or two doses, respectively (fold changes vs control group >1.5) (Fig. 5B). While the number of upregulated genes with REGN3767 treatment kept increasing (131 genes after one dose vs 731 genes after two doses), the number of cemiplimab induced genes decreased (696 upregulated genes after one dose vs 161 genes after two doses), indicating different kinetics of response to these monotherapies. Transcriptional gene expression profiles indicative of different TILs subpopulations in the combination group showed the enrichment of immune cell signatures, including neutrophils, macrophages, NK and T cells; all signatures were more pronounced after two doses (Fig. 5C). The combination therapy also enhanced immune responses promoted by either antibody alone, including genes associated with T cells activation and effector function. In the T cells signature, a prominent increase in the expression of Cd40lg receptor, which is involved in costimulatory T cell receptor signaling, was observed in a combination treatment (Fig. 5C). Blocking PD-1 and LAG-3 in combination therapy also resulted in upregulation of inhibitory PD-L1 and VISTA ligands that can suppress T cell responses, suggesting a potential mechanism of adaptive immune escape (Fig. 5D). Cytotoxic Gzma, Gzmb and Prf1 as well as selected T cell immunoregulatory and Th1 signaling genes were upregulated in the combination treatment group, consistent with our finding that combination treatment enhanced the cytotoxic function of intratumoral T cells after tumor antigen re-stimulation (Fig. 3C).
Pharmacokinetics and toxicology studies of REGN3767 in cynomolgus monkeys

Twenty-five (25) female cynomolgus monkeys (5 animals/dose group) received a single i.v. infusion (1, 5, or 15 mg/kg) or s.c. injection (1 or 15 mg/kg) of REGN3767 and animals were followed over the 8-week study period. Single dose PK of functional REGN3767 showed linear kinetics with a half-life of 10-12 days (Fig. 6). The immunogenicity of REGN3767 was high, with 5/15 and 5/10 animals showing positive ADA responses following i.v. and s.c. administrations, respectively, but there were no observable adverse effects. Following i.v. infusion of REGN3767, the PK parameters were estimated using noncompartmental analysis (NCA) (Supplementary Table S2). The mean C\text{max} increased in an approximately dose-proportional manner across the tested dose levels. Following i.v. infusion of REGN3767, the mean AUC\text{inf} increased in a dose-proportional manner as demonstrated by similar AUC\text{inf}/dose values. Consistent with this finding, the mean clearance (CL) values were similar for the 3 dose groups, ranging from 4.11 to 4.47 mL/day/kg. The elimination phase t\text{1/2} values for the 3 dose groups were also similar, ranging from 10.8 to 11.5 days.

In a cynomolgus monkey toxicology study, 5 animals/sex/group were given 0, 2, 10, or 50 mg/kg/week REGN3767 by i.v. infusion for 4 consecutive weeks followed by an 8-week recovery period. REGN3767 was well tolerated at all dose levels. There were no unscheduled deaths during the study and no drug-related clinical signs evident. There were no REGN3767-related effects on body weights, body temperatures, clinical pathology, cardiovascular, or neurological endpoints, with no substantial sex differences noted. In addition, there were no REGN3767-related changes in lymphocyte subpopulations in PBMC, organ weights, or findings from the gross and microscopic examination of tissues from
primary necropsy (day 30) or after the 8-week recovery period (day 85). The no
observable adverse effect level (NOAEL) for this study was considered to be 50 mg/kg, the
highest dose level evaluated.

DISCUSSION

Blockade of inhibitory pathways on activated T cells with mAbs against the immune
checkpoint receptors PD-1 and CTLA-4 has shown remarkable antitumor activity in diverse
human cancers. Further, therapeutic co-targeting of PD-1 and CTLA-4 showed enhanced
clinical activity compared to single agents. These successes have led to the exploration of
additional inhibitory signaling pathways on immune cells. LAG-3 receptor is another
inhibitory checkpoint on T cells; its potential role in cancer is supported by extensive
preclinical studies (8, 22, 23). Notably, analysis of mouse and human tumors suggests that
co-expression of LAG-3 and PD-1 is associated with T cell dysfunction (21, 22), indicating
that inducible LAG-3 expression on activated T cells could be a potential mechanism of
therapeutic resistance to PD-1 checkpoint blockade (46). These findings have prompted
efforts to explore using anti-LAG-3 blocking antibodies, either alone or in combination with
anti-PD-1, in cancers (32-34).

To target LAG-3, we generated a human mAb REGN3767 that binds to human and monkey
LAG-3 and inhibits the interaction with its ligand, MHC class II. REGN3767 is engineered as
human IgG4 isotype and contains one mutation in the hinge region to minimize half-
antibody formation and three additional point mutations in Fc-region to reduce the
potential for inducing antibody dependent cytotoxicity against LAG-3 expressing cells. We
confirmed that REGN3767 does not elicit antibody Fc-mediated ADCC or CDC activity. In
Characterization of the Anti-LAG-3 Antibody REGN3767

cell-based assays, REGN3767 rescued T cell activation as a single agent and enhanced responses to a clinical anti-PD-1 antibody, cemiplimab, demonstrating that LAG-3 delivers an independent inhibitory signal to activated T cells during PD-1 blockade. In an engineered T cell/APC bioassay, the combination of REGN3767 and cemiplimab, was able to overcome the inhibitory effects of MHC II/LAG-3 and PD-L1/PD-1 signaling. In a second assay, REGN3767 was able to enhance the response to cemiplimab of primary CD4+ T cells in allogeneic T cell:PBMC MLR assay.

Consistent with in vitro findings, REGN3767 therapy reduced tumor growth in a subset of human PD-1XLAG-3 knock-in mice, and improved anti-tumor efficacy of cemiplimab, thus providing preclinical proof of concept for dual PD-1 and LAG-3 blockade in cancer patients. The potential mechanism underlying the additive effect of REGN3767 and cemiplimab was consistent with T cell activation both in tumors and the periphery of MC38.Ova bearing mice. Combination treatment was associated with increased intratumoral CD4+ and CD8+ T cells producing IFNγ, TNFα, as well as elevated IFNγ, TNFα and IL-10 levels in blood and spleen. Although IL-10 is normally associated with reduced antitumor activity (47), some reports describe a role of IL-10 in expanding tumor CD8+ T cells (48).

The role of REGN3767 in modulating T cell responses was further supported by robust transcriptional changes consistent with T cell expansion and activation in tumors in response to REGN3767 therapy. Combination therapy elicited additional immune-related gene changes, not seen with either antibody alone. Surprisingly, in addition to T cell expansion and activation, REGN3767 therapy engaged other types of tumor-associated leukocytes, including macrophages, neutrophils and NK cells, suggesting that the myeloid compartment may contribute to the therapeutic response following anti-LAG-3 therapy. The physiological role of LAG-3 on immune cell types other than T cells, including
Characterization of the Anti-LAG-3 Antibody REGN3767

plasmacytoid DCs, natural killer cells, and B cells is poorly understood (8, 10), and may be related to additional LAG-3 ligands reported recently. It has been suggested that LSECtin, a member of the DC-SIGN family, and Galectin-3, both expressed in many tumors, are ligands that can also regulate LAG-3 expressing CD8+ T cell and NK cells in tumor microenvironment (49, 50). Fibrinogen-like protein FGL1, a liver-secreted protein, is another LAG-3 functional ligand independent of MHC-II (51). At this time, it is not clear how REGN3767 affects LAG-3 interaction with additional putative ligands. Detailed studies as to when and where these putative ligands are expressed and how they interact with LAG-3 will further facilitate unravelling the mechanisms of LAG-3 targeting.

Neither REGN3767 nor cemiplimab, as monotherapy or in combination caused non-specific lymphocyte activation in an ex vivo cytokine release assay. Cynomolgus monkey toxicology studies were performed separately for REGN3767 and cemiplimab and there were no potential adverse effects related to lymphocyte-binding patterns of these antibodies. Our results expand on the existing preclinical data that combination blockade of PD-1 and LAG-3 signaling has potential for increased therapeutic benefit in cancer treatment. REGN3767 in combination with cemiplimab is currently being investigated clinically in multiple tumor types.

Disclosure of Potential Conflicts of Interest
Regeneron Pharmaceuticals, Inc. is developing REGN3767 as a clinical compound. All authors are employees of Regeneron Pharmaceuticals, Inc. Regeneron Pharmaceuticals, Inc. has filed patent applications related to this work for REGN3767 and E. Ullman, A. Hermann, E. Ioffe, E. Burova, G. Thurston are inventors on patent application (#20170101472).

Authors’ Contributions
Characterization of the Anti-LAG-3 Antibody REGN3767

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** E. Burova, A. Hermann, J. Dai, E. Ullman, G. Halasz, T. Potocky, S. Hong, M. Liu, O. Allbritton, A. Woodruff, J. Pei, A. Rafique

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** E. Burova, A. Hermann, J. Dai, E. Ullman, G. Halasz, T. Potocky, S. Hong, M. Liu, A. Rafique, J. Martin, D. MacDonald, W. Olson, A. Murphy, E. Ioffe, G. Thurston, M. Mohrs

**Writing, review, and/or revision of the manuscript:** E. Burova, E. Ioffe, G. Thurston, M. Mohrs

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** E. Burova, A. Hermann, J. Dai, E. Ullman, G. Halasz, T. Potocky, S. Hong, M. Liu, O. Allbritton, A. Woodruff, J. Pei, A. Rafique

**Study supervision:** E. Burova, A. Hermann, J. Dai, J. Martin, W. Olson, A. Murphy, E. Ioffe, G. Thurston, M. Mohrs

**Acknowledgments**
The authors thank all Regeneron employees who contributed to the generation and characterization of REGN3767 including R. Leidich, A. Badithe, P. Kruger, C.-J. Siao, A. Mujica, H. Polites, G. Kroog, I. Lowy, and N. Papadopoulos.
Characterization of the Anti-LAG-3 Antibody REGN3767

REFERENCES


Characterization of the Anti-LAG-3 Antibody REGN3767


Characterization of the Anti-LAG-3 Antibody REGN3767


Characterization of the Anti-LAG-3 Antibody REGN3767


Characterization of the Anti-LAG-3 Antibody REGN3767


Characterization of the Anti-LAG-3 Antibody REGN3767


43. Hannier S, Triebel F. The MHC class II ligand lymphocyte activation gene-3 is co-distributed with CD8 and CD3-TCR molecules after their engagement by mAb or peptide-MHC class I complexes. Int Immunol 1999;11:1745-52.


Characterization of the Anti-LAG-3 Antibody REGN3767


Figure 1. Binding characteristics and in vitro activity of anti-human LAG-3 antibody REGN3767. A, REGN3767 displays dose-dependent binding to HEK293 cells expressing human or cynomolgus monkey (mf) LAG-3. HEK293 wt (left figure), HEK293/hLAG-3 (middle figure) and HEK293/mfLAG-3 (right figure) cells were incubated with REGN3767 (black circles) or isotype control mAb REGN2759 (grey triangles) pre-complexed with Alexa 647 Fab anti-hIgG. The x-axis indicates the antibody \( \text{Log}_{10} \) concentration and the y-axis indicates the geometric median fluorescent intensity (MFI) of Alexa 647 cell staining. B, REGN3767 antibody blocks adherence of Raji and A20 cells expressing endogenous MHC II to HEK293 cells expressing human LAG-3. Raji cells or A20 cells fluorescently labeled with Calcein AM were added to HEK293/hLAG-3 or HEK293 wt cells that had been pre-treated with anti-LAG-3 Ab REGN3767 (black circles) or isotype control REGN2759 Ab (grey triangles). The x-axis indicates the antibody \( \text{Log}_{10} \) concentration and the y-axis indicates the fluorescence intensity given in relative fluorescence units (RFU). C, Combination blockade with cemiplimab and REGN3767 enhances CD4\(^+\) T cell function in an allogeneic MLR. IFN\(\gamma\) secretion in culture was assayed after coculturing \(10^5\) CD4\(^+\) T cells (initially exposed to PBMCs) with freshly mitomycin C treated \(2 \times 10^5\) PBMCs with or without test antibodies for 4 days.

Figure 2. Anti-tumor responses of cemiplimab and REGN3767 combination treatment in MC38.Ova tumor model in human PD-1xLAG-3 knock-in mice. A and C, Prophylactic cemiplimab+REGN3767 treatment inhibits MC38.Ova tumor growth. On day 0, human PD-1xLAG-3 knock-in mice were injected s.c. with \(5 \times 10^5\) MC38.Ova cells and randomized into four treatment groups. Mice were administered REGN3767 (25 mg/kg;
Characterization of the Anti-LAG-3 Antibody REGN3767

N=12), cemiplimab (10 mg/kg; N=12), REGN3767 (25 mg/kg) + cemiplimab (10 mg/kg) combination (N=12), or hIgG4 control (25 mg/kg; N=6) by i.p. injection on days 3, 7, 10, 14 and 17. A, Average tumor volumes (mm$^3$ ± SEM) in each treatment group. Treatment days are indicated by arrows (upper left graph). Individual tumor volumes in each treatment group were measured on day 22, the last time point when all animals in the study were alive (upper right graph). Statistical significance was determined by one-way ANOVA with Tukey’s multiple comparison post-test (*p<0.05; ** p<0.01). Kaplan-Meier survival curves of mice treated with single agents or combination agents are shown (bottom graph). A log-rank (Mantel-Cox) test revealed that combination therapy or cemiplimab monotherapy significantly prolonged mouse survival compared to the control group (**p< 0.0001 and **p< 0.01, respectively). C, Individual tumor growth curves in each treatment group. The number of tumor-free (TF) animals on day 32 is shown. B and D, Cemiplimab + REGN3767 combination treatment delays growth of established tumors. PD-1×LAG-3 knock-in mice were injected s.c. with 5×10$^5$ MC38.Ova cells on day 0, randomized into four treatment groups on day 10 (108 mm$^3$ mean tumor volume per group) and treated by i.p. injection with cemiplimab (10 mg/kg; N=10), REGN3767 (25 mg/kg; N=9), combination cemiplimab + REGN3767 (10 mg/kg + 25 mg/kg; N=11) or isotype control antibody (25 mg/kg, N=7) on days 10, 14, 17, 22 and 25. B, Average tumor volumes (mm$^3$+/ SEM) in each treatment group (left graph). Individual tumor volumes in each treatment group were measured on day 22, the last time point when all animals in the study were alive (right graph). D, Individual tumor growth curves in each treatment group.

Figure 3. Combination cemiplimab and REGN3767 treatment results in enhanced cytokine production by tumor-infiltrating T cells. 5×10$^5$ MC38.Ova cells were injected
Characterization of the Anti-LAG-3 Antibody REGN3767

s.c. into female human PD-1xLAG-3 knock-in mice. At day 11 post implantation, mice were randomized into four treatment groups with an average tumors size of 100 mm³ (n=12 per group). Mice were dosed i.p. either with hIgG4 control antibody (25 mg/kg), or cemiplimab (10 mg/kg) or REGN3767 (25 mg/kg), or combination of cemiplimab and REGN3767 (10 mg/kg and 25 mg/kg, respectively). Antibodies were administered on days 11 and 15, and on day 18 post implantation tumors were harvested, dissociated into single cell suspensions and stained for flow cytometry. Data are representative of two combined experiments. 

A, Individual tumor volumes in each treatment group were measured on day 18 at harvest. 

B, Numbers of CD4⁺ and CD8⁺ T cells within tumors after treatments on day 18. Tumors were dissociated into single cell suspension, CD45-enriched fraction was isolated through Percoll gradient centrifugation and further processed for flow cytometry. 

C, Plots of IFNγ⁺ or TNFα⁺ cells of total tumor-infiltrating CD8⁺ or CD4⁺ T cells, following ex vivo OVA MHC I and MHC II peptides stimulation. 

D, Plots of IFNγ⁺ cells of total draining lymph node CD4⁺ and CD8⁺ T cells. Tumor inguinal dLNs were isolated and stimulated with PMA/ ionomycin for 4 hours in the presence of Brefeldin A, then analyzed for cytokine production by intracellular staining and flow cytometry. Error bars depict SEM. Nonparametric one-way ANOVA with Kruskal-Wallis test was used with Dunn's post-test (⁎ p<0.05, ** p<0.01, **** p<0.0001).

Figure 4. Combined cemiplimab and REGN3767 treatment results in enhancement of cytokine secretion in serum and spleens by therapeutic antibodies. PD-1xLAG-3 knock-in mice were injected s.c. with 5x10⁵ MC38.Ova cells. At day 11 post implantation, mice were randomized into treatment groups with an average tumor size of 100 mm³. Mice were dosed i.p. either with isotype control antibody (hIgG4) at 25 mg/kg, cemiplimab at 10
Characterization of the Anti-LAG-3 Antibody REGN3767

mg/kg, REGN3767 at 25 mg/kg, or the combination of cemiplimab and REGN3767 at 10 mg/kg and 25 mg/kg, respectively. Antibodies were administered on days 11 and 15, and on day 18 post implantation serum and spleens were collected. Spleens were dissociated into single cell suspensions. Levels of serum and spleen cytokines were measured with V-PLEX Proinflammatory Panel 1 mouse kit (Meso Scale Discovery). Data points represent the medians plotted in a box-and-whiskers plots (Prizm software) of the cytokines levels in serum (A) and spleens (B) in each treatment group at collection time point. Results are shown for IFNγ, TNFα and IL-10.

Figure 5. Changes in expression of immune-related genes in tumors treated with cemiplimab, REGN3767, and the combination.

A, Mean volumes of MC38.Ova tumors in PD-1xLAG-3 mice, treated with single dose or two doses of control antibodies, cemiplimab, REGN3767, or cemiplimab+REGN3767. On day 0, MC38.Ova cells (5x10^5 cells/mouse) were implanted subcutaneously and mice were randomized on day 10, when tumors reached 100 mm^3. One group of mice was treated on randomization day 10 with a single dose of control antibodies, REGN3767, cemiplimab or their combination and another group was treated with two doses (on day 10 and day 14). Tumors in a single dose treatment group were harvested on day 14 and tumors in two doses treatment group were harvested on day 17. Data represent analysis of 9-10 mice per each antibody treatment, mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001. B, Differentially expressed genes with at least 1.5 fold change by RNA seq analysis after single or two doses of single or dual blockade of cemiplimab and REGN3767. C, Heat map of pre-defined marker genes for neutrophils, macrophages, NK cells, T cells and cytokines/cytotoxic genes regulated by combination therapy. For single or two doses points, fold changes relative to
Characterization of the Anti-LAG-3 Antibody REGN3767

median values for the isotype controls are shown. D, Combination treatment results in increased relative levels of mPD-L1 and mVISTA RNA (normalized to mouse cyclophilin RNA) compared to the single antibody or isotype control group (assigned a value of 1.0). Mice were treated as in A, and tumors were collected after single or two doses of monotherapy or dual therapy of cemiplimab and REGN3767. Tumor RNA was analyzed by Taqman real-time PCR. Values are median plotted in box-and-whiskers plot with whiskers extended from minimum to maximum. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. Pharmacokinetic parameters for REGN3767 following single i.v. and s.c. administration to cynomolgus monkeys. Single dose PK of REGN3767 in cynomolgus monkeys shows linear kinetics with a half-life of 10-12 days. Anti-drug antibody (ADA) impacted REGN3767 concentrations in 33% (5/15) and 50% (5/10) of i.v. and s.c. group animals, respectively. ADA-impacted concentrations were excluded. Abbreviations: LLOQ, lower limit of quantification.
**Figure 1**

A. HEK293 wt, HEK293/hLAG-3, HEK293/mfLAG-3

B. Raji cells (express human MHC II), A20 cells (express mouse MHC II)

C. Donor 1, Donor 2
Figure 2

A

B

C

D

hlgG4
REGN3767
REGN2810
REGN2810+REGN3767

% Survival

0
10
20
30
40
50

Day 22

Day 22

hlgG4
0/6 TF

REGN2810
2/12 TF

REGN3767
1/12 TF

REGN2810

REGN2810+REGN3767

REGN3767

REGN2810+REGN3767

Tumor Volume, mm³

Tumor Volume, mm³

Tumor Volume, mm³

Tumor Volume, mm³

Time after inoculation (d)

Time after inoculation (d)

Time after inoculation (d)

Time after inoculation (d)
Figure 3

(A) Tumor Size (mm³) for different treatments:
- cemiplimab
- REGN3767

(B) Cell Numbers / mm³ for different treatments:
- CD4⁺ T
- CD8⁺ T
- IgG4
- cemiplimab
- REGN3767
- cemiplimab + REGN3767

(C) % IFN-γ⁺ of CD4⁺ for different treatments:
- cemiplimab
- REGN3767

(D) % IFN-γ⁺ of CD8⁺ for different treatments:
- cemiplimab
- REGN3767

* Significant differences
** Highly significant differences
**** Extremely highly significant differences
**Figure 4**

(A)

(B)

Graphs showing cytokine production: IFN-γ, TNF-α, IL-10, and IL-12 levels in response to different treatments. The graphs compare untreated control (black bars), hIgG4, cemiplimab, REGN3767, and combinations, with levels of cytokines measured in pg/ml. Statistical significance is indicated by asterisks: **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 5

A

**Single Dose**

**Two Doses**

B

**number of gene changes**

**Tumor Volume, mm³**

C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor volume at collection</th>
<th>Tumor fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>REGN2810</td>
<td>0</td>
<td>REGN2810</td>
</tr>
<tr>
<td>REGN3767</td>
<td>5</td>
<td>REGN3767</td>
</tr>
<tr>
<td>REGN2810+REGN3767</td>
<td>10</td>
<td>REGN2810+REGN3767</td>
</tr>
</tbody>
</table>

**Tumor fold change**

D

**PD-L1**

**VISTA**

**Relative RNA Level**

Downloaded from mct.aacrjournals.org on May 18, 2021. © 2019 American Association for Cancer Research.
Preclinical Development of the Anti-LAG-3 Antibody REGN3767: Characterization and Activity in Combination with the Anti-PD-1 Antibody Cemiplimab in Human PD-1xLAG-3 Knock-In Mice

Elena Burova, Aynur Hermann, Jie Dai, et al.

*Mol Cancer Ther* Published OnlineFirst August 8, 2019.

**Updated version**
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-18-1376

**Supplementary Material**
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2019/08/08/1535-7163.MCT-18-1376.DC1

**Author Manuscript**
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/early/2019/08/08/1535-7163.MCT-18-1376. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.